Analyzing Neuronal Connectivity through Conditioned Taste Aversion in rats

Yuval Bonneh

205735194

Sagol School of Neuroscience, Tel-Aviv University

PI: Dr. Anan Moran

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Abstract

Neural connectivity has been studied since the early 1950s. While this field of study evolved through different levels of focus and methods of analysis, starting from single connections and post-mortem operations to using electrophysiology and novel probe technology along with computed data analysis, it can still be improved to be more accurate. In this study, we aimed to examine cross-correlation analysis methods on data from free-behaving animals to estimate neural connectivity within a neuron population following a behavioral procedure. To do so, we applied principles from Kobayashi's Generalized Linear Model Cross-Correlation (GLMCC) method on data from free-behaving rats. The data was extracted from one female rat's gustatory cortex and adjacent somatosensory areas of the brain using NeuroPixel probe, following a procedure that led to a conditioned taste aversion, a powerful model for studying learning and memory processes in the brain. In accordance with what we know about long-term memory formation, our results revealed dynamic patterns of connectivity over time during memory acquisition (0-3 hours) and consolidation (5-7 hours) stages. In the memory acquisition phase, we noticed an increase in the number of excitatory connections in the gustatory cortex, while in the consolidation phase the connections seemed to be stronger overall. Moreover, excitatory- and inhibitory-oriented neurons show different patterns of activity, as well as neurons in different brain regions, suggesting there are different roles in filtering and processing the information. The GLMCC method proved to be effective in differentiating true connections from the effects of surrounding neurons, providing a robust analysis of connectivity in neuron ensembles. However, this study is a preliminary work which focused on the technical parts of the analysis method and therefore lacks some important factors. First, it only shows how network activity changes over time in a small part of the brain of one rat. Second, it is only looking into the first hours following a behavioral process, while the network activity before and longer after this process could add important information. Future studies should include larger samples, and analyze data from longer time periods before, during and after the behavioral process, as well as recording data from other parts of the brain. In those cases, another factor we must address is that neurons from different parts of the brain may have different properties that might affect how we analyze the data, and therefore require different calculations and parameters blended in to the GLMCC method. Overall, this study opens avenues for further research into the complexities of brain network connectivity and its role in learning and memory processes.

Introduction

Memory is an essential cognitive function that allows us to store and retrieve information about past events, experiences and knowledge. Together with Learning processes, they are two key elements that are crucial for survival. For instance, an animal must be able to identify its predators and avoid them once their presence is sensed in order to stay alive. To do so, the animal must *learn* the danger signs, *remember* that information, and translate that knowledge to *behavior*. The brain is an intricate organ that plays a vital role in every step of the way.

There are two primary forms of memory: short-term memory and long-term memory. As the first relates to the ability to hold and manipulate information for brief periods of time, the latter refers to the ability to store and retrieve complex information gathered over time, and therefore it is more crucial for learning processes[1]. The formation of long-term memories is based on changes in the excitability and synaptic connections between neurons as they evolve through different stages, such as memory acquisition and consolidation[19]. Consolidation is the process of stabilizing a memory after it is formed, and it is thought to involve the transfer of information from the hippocampus to the neocortex[2]. Some mechanisms that are known to control these changes are long-term potentiation (LTP) and long-term depression (LTD)[18]. These two reverse processes describe fluctuations in synaptic strength, which are related to N-methyl-D-aspartate receptors (NMDAR) and $Ca2^{+}$ flux into the postsynaptic cell as well as protein synthesis. Recent evidence shows that the LTP-LDP crossover point is NMDAR dependent, meaning that increased NMDAR activity possibly leads to LTP, and the other way around for LTD[20]. While these two rules explain a major part of memory consolidation, there are still many unanswered questions regarding this process. First, LTP and LTD are relevant mostly in the molecular, single-neuron level, but the mechanisms that govern this process in the network level, as well as the role that each neuron plays in a network, are still to be uncovered. Second, the temporal manner of neuronal changes in excitability and connectivity remains unclear. Moreover, most research in this field was done in hippocampal-cortical pathways whereas other pathways in the brain may show different mechanisms.

Conditioned taste aversion (CTA)[6] is a powerful form of learning by classical conditioning that occurs when an animal associates a specific taste with an aversive outcome, such as nausea or illness, even after just one pairing[7]. The neurological mechanism of this process mainly involves the basolateral amygdala (BLA) and the gustatory cortex (GC)[9]. In addition, biochemical studies of the GC during CTA have identified two distinct memory phases in the hours after training: an acquisition phase that takes place during approximately the first 3 post-training hours[22] and a consolidation phase that occurs approximately 5–7 h post-training[23]. More recent research has shown that following CTA in alert rats, neuronal activity patterns increase only during these two phases, and change differently for different neuron organization levels of the process[21].

In the early years of CTA study researchers used behavioral analysis and lesions to investigate the process and the brain regions involved[11,13]. Over the years more accurate methods such as calcium imaging[14], immunostaining and Fos-like immunoreactivity(FIL)[10] have developed and yielded decent, yet uncertain

results[11]. While some researchers seek to investigate the molecular basis of this process[15], others look into the whole brain activity involved in this process and how it is interconnected[10,13,15]. By exploring the neural mechanisms behind CTA on animals in-vivo, researchers hope to gain a better understanding on how neuron networks function and rewire in the context of learning and memory.

Neuron ensembles were first proposed as a focus for research on brain mechanisms of learning by Donald Hebb in 1949[12]. He suggested that specific external stimuli selectively activate specific patterns of neurons that interact with each other in different brain regions. Since then, this field of research have developed and methods such as electrophysiology and cellular imaging supported the claim that learned associations between environmental cues and their outcome (such as reward or malaise) are encoded in neuronal ensembles that are activated by both stimuli. Hence, the neuron-ensemble hypothesis has been an important part of behavioral research in animals until present days[11].

This kind of research, using modern data-acquisition techniques as mentioned, also requires the ability to conduct data analysis. In order to examine connection properties of neuronal population, one must look into the correlations between the neurons, also referred to as Cross-Correlation (CC), as evaluated by Aertsen & Gerstein in 1985[26]. Correlation is a measure of the degree to which trial-to-trial fluctuations in response strength are shared by a pair of neurons, or in other words – the degree to which one neuron is influenced (and vice versa) by the firing of another neuron from which the first neuron is receiving input[16]. This method also allows reducing signal-to-noise ratio, and measure properties such as firing rate(FR), tuning curve, bursting factor and other properties of a single neuron as well as neuron populations. CC methods have been used widely to investigate neuronal connectivity in the past few decades[17, 24-28]. One method by Spark et al.,[24] claims that calculating the CC between spike trains is not as accurate as it gets, depending on neuron properties. Spark suggests using deconvolution, a mathematical method used in image processing, to decompose the signal into elements and remove the effects of non-transmission patterns such as fire rate and bursting. Another recent model of analyzing connectivity with CC is Kobayashi's General Linear Model Cross-Correlation (GLMCC)[17]. This method uses CC between neuron spike trains, as well as statistical and mathematical functions to estimate neuronal connectivity in a neuron ensemble.

This research aims to examine neuron ensemble analysis methods by operating CC-based estimations of network connectivity of a neuron population while also focusing on different properties of the given data.

Methods

Surgical procedures and Cognitive Taste Aversion paradigm

The data in this research was obtained from prior lab work and includes spike-trains of 132 neurons in rat's GC and S2 brain regions from the first 16 hours post-CTA. Recording was made using NeuroPixel probe. In addition, an intraoral cannula (IOC; flexible plastic tubes capable of conveying

liquid) was cemented to the rat inside cheek in order to control the rat's gustatory stimuli. To induce CTA, an oral injection of sucrose solution (Suc, 0.5M) via the IOC was followed by an injection of Lithium Chloride (LiCl. 0.15M, 2% body weight), a substance known to cause malaise[8]. Therefore the rat associates the taste of sucrose with malaise and develops CTA. In this project we investigated methods for estimating connectivity in neuron ensembles by calculating cross-correlation between neurons.

Recording and data acquisition

NeuroPixel

A state-of-the-art technology for large-scale electrophysiological recording. Based on active silicone, this tool is designed for high spatial and temporal resolution recording from neurons. Each probe has 384 recording channels that are programed to address 960 processing sites simultaneously, while only occupying a single 10-mm long, 70 × 20-µm cross-section shank in size (Fig. 1). The fully integrated functionality and small size of NeuroPixel probes allows recording large populations of neurons, from several brain structures in freely moving animals simultaneously and for long periods of time[25]. This opens the way for research in neural mechanisms of behavior in animals.

Data recorded with NeuroPixel probes was automatically spike-sorted with Kilosort software. By comparing each spike's properties (such as amplitude, waveform consistency, and the presence of short-latency ISIs) to its neighbors in each time point, the team could determine whether similar spikes should be clustered into one '*good unit' (GU)*, while also excluding spikes that does not match with its neighbors or meet the criteria of a normal functioning neuron and categorizing them as *'multi-unit-activity'* or noise. The final product of this stage is temporal spike-train that includes time of spike (in ms) and cluster ID for each clustered GU, which we considered to be a single neuron.

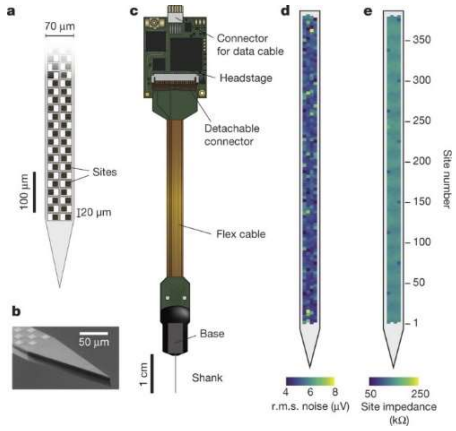


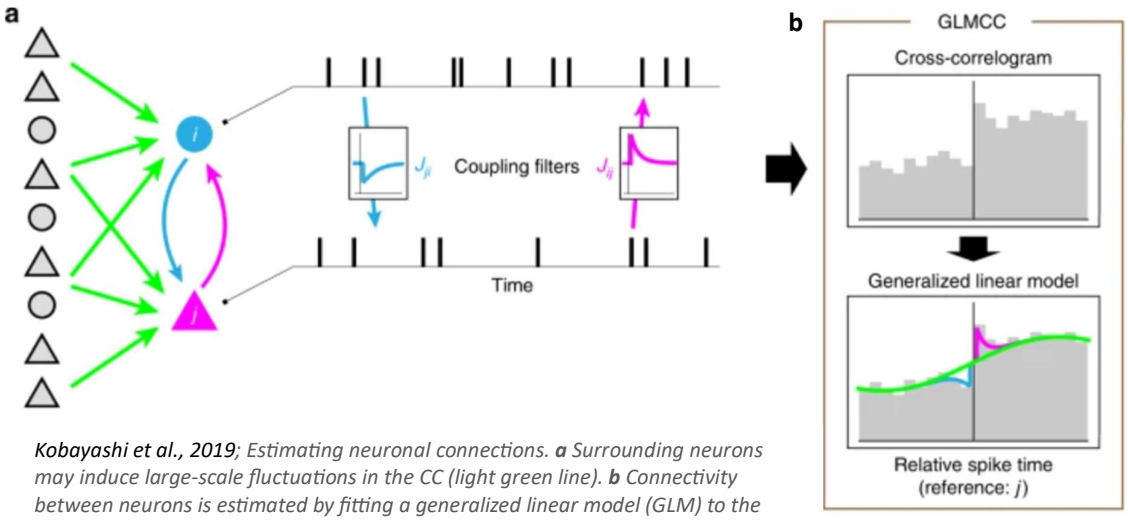
Figure 1:
*Jun, J. J. Et al, 2017; **a**, Illustration of probe tip, showing checkerboard site layout (dark squares). **b**, Scanning electron microscope image of probe tip. **c**, Probe packaging, including flex cable and headstage for bidirectional data transmission. **d**, Example of r.m.s. noise levels of the AP band in saline, for 384 sites (switchable option). Mean ± s.d. = 5.1 ± 0.6 µV. **e**, Typical site impedance in saline, for 384 sites, measured for each site with sinusoidal 1 nA injected currents at 1 kHz (see Methods). Mean ± s.d. = 149 ± 6 kΩ (Jun, J., Steinmetz, N., Siegle, J. et al., 2017)*

Cross-Correlation Histograms(CCH) and Generalized Linear Model

CCHs are a form of viewing the temporal proximity between two neurons' activity, where bin width represents a timeframe (usually a few milliseconds), and bin height shows the number of times a target neuron (i) fired temporally relative to the firing of the reference neuron (j) spike, represented as the setpoint line (0). Thus, bins that are close to the set point represent spikes that occurred temporally closer to the reference neuron firing, positive bins represent spikes that came after the reference neuron spike, and negative bins stand for spikes that came before the reference neuron spike.

Fig. 2b (top) taken from Kobayashi's article[17] shows a simple example of one CCH, in which there are higher bins in the positive half, and thus more spikes of the target neuron (i) after spikes of the reference neuron (j) than we might see by calculating the expected spiking activity. This could imply that there is a link between the firing of reference neuron j and the consequent firing of target neuron i and therefore an excitatory connection from neuron j directed to neuron i. Moreover, from this example we could also assume an inhibitory connection the other way around from target neuron i to reference neuron j, since there are less spikes of neuron i just before each spike of neuron j, then we might expect in a non-connection situation. That being said, these assumptions have to be affirmed statisticly.

Figure 2:



*Kobayashi et al., 2019; Estimating neuronal connections. **a** Surrounding neurons may induce large-scale fluctuations in the CC (light green line). **b** Connectivity between neurons is estimated by fitting a generalized linear model (GLM) to the cross-correlation (CC). J_{ij} represents a coupling from the j-th neuron to the i-th neuron. Excitatory and inhibitory neurons are depicted as triangles and circles, and their synaptic connections are colored magenta and cyan, respectively.*

To do so, we used Kobayashi's Generalized Linear Model Cross-Correlation (GLMCC) method. This method seeks to prove monosynaptic connectivity between two neurons by looking at the first few milliseconds before and after the setpoint (0) in the CCH. Each spike of the neuron pair could either be related to the other neuron's spike, or to one of the neighboring neurons. Thus, summing up all temporally related spikes within 50 ms between a pair of neurons results in a histogram that shows temporally relativeness but also contains the effect of other neurons in the area (Fig. 2a). By applying a GLM statistic tool on each histogram, according to Kobayashi, we generate a figure of large-scale slow wavy fluctuations that depicts the general effects of surrounding neurons on the two-neuron histogram (Green line, Fig. 2b). This line also represents the null hypothesis that there is no connection between the two neurons. Therefore, any deviation from that line which is statistically significant could reject the null hypothesis and claim that there is in fact a direct connection.

To calculate the possible deviation, coupling filters are used to calculate the excluded area in the histogram below or above the green line within a several milliseconds from the setpoint in both directions. This value is considered to be Post-synaptic Potential (PSP) and it is used as the measurement value for neuronal connectivity (Fig. 2b; magenta marks EPSP, cyan is IPSP, green line is expected activity under no-connection). Note that neurons are marked as excitatory (magenta) or inhibitory (cyan) as a preliminary status, that doesn't imply they are in fact purely excitatory or inhibitory neurons. We refer only to the specific connection between two neurons to be excitatory or inhibitory in the same manner described in the Figure, while later summing up excitatory and inhibitory connections of each neuron with all other neurons to decide whether it is an excitatory- or inhibitory-oriented neuron accordingly.

The existance of a connection between pairs of neurons is determined by a confidence level. Here we used Kobayashi's recommended confidence level of $\alpha = 0.001$ to determine true connectivity, avoiding as many false positives (FP) and false negatives (FN) as possible. Kobayashi's conclusion regarding significance was a result of simulating a-1000 neuron system and defining the firing patterns of excitatory (80%) and inhibitory neurons (20%), then testing the GLMCC method for different significance levels and comparing it to true connectivity. The α that best resembled true connectivity was $\alpha = 0.001$[17].

Connectivity Matrix

Here we used python interface to generate the CCH in all pairs of the whole neuron ensemble (size N) by comparing every two spike-trains with 50 ms intervals before and after the setpoint (see Fig 3 – results). The process included iterating through all spike-trains, comparing every spike-train to all

other spike-trains which it was yet to be compared to, while also operating GLMCC functions on the received CCH to calculate the significant, directional PSP values between each pair of neurons. We also produced a CCH for each pair of neurons examined and wrote the significant PSP values (J_{ij}) in the corresponding junction (ij) of the *connectivity matrix* W (two-dimensional N x N matrix), which was exported to '.csv' file.

In further analysis we used gathered and known information about the units to divide the matrix into four subgroups; initially, we used the results of the first part to calculate the amount of excitatory and inhibitory (E/I) connections for every neuron (every column in the matrix) and divide the the group into excitatory- and inhibitory-oriented units (excitatory first, e.g., units 0-12 excitatory, 13-20 inhibitory). Then, we matched each unit to its known location on the probe to either GC or S2, with a threshold of 1200μm (GC first, e.g., Units 0-25. Those below the threshold are GC units and those above are S2 units, before dividing each of the two groups individually into E/I subgroups. The result is four groups of neurons: GC/E, GC/I, S2/E, S2/I – and units were repositioned in the matrix in that order 0 – N, and aligned against each other in a new connectivity matrix (size N x N). The threshold was decided based on where the units' characteristic activity patterns roughly differ. In our data the major alteration occurred near the depth of 1200μm (probe channel depths are marked 0 at the tip and 3840μm at the root; see Fig. 1)

The python scripts that were used through these steps are self-generated, but deeply inspired by Kobayashi's main 'glmcc' script. The final product is available in Github (https://github.com/Yuvalb94/cross-correlation-project-YB).

Results

We investigated pre-acquired data from one female rat in the first 16 hours post CTA administration. The data included spike-trains from 132 distinct units (neurons).

Cross-Correlation analysis with GLMCC was made for each pair of neurons. Figure 3 shows two of the CCH we obtained. A significant connection is marked by magenta (excitatory) or cyan (inhibitory) lines which calculate exclusions from the expected spike-count (green line). Connections that appear on the right half of the histogram are considered to be originated from the reference neuron (j) and headed to the target neuron (i), since the spike-count up to five miliseconds after the setpoint can be considered a direct effect of the reference neuron (j) spikes. Connections that appear on the left half of the histogram are considered to be originated from the target neuron (i) and

headed to the reference neuron (j), since the spike-count up to five miliseconds before the setpoint (0) could have the greatest effect (excitatory or inhibitory) on the reference neuron. The PSP is considered to be the area beteeen the magenta/cyan lines and the green line, and it is the value in the relevant junction in the connectivity matrix.

Figure 3

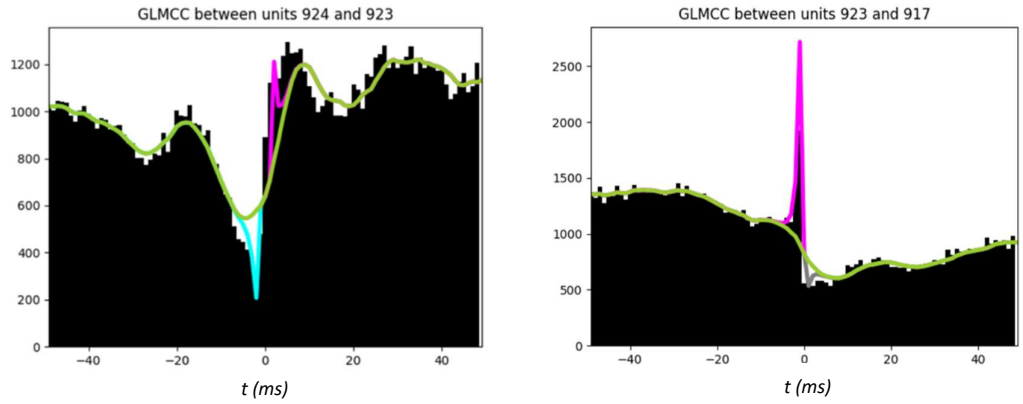


Fig.3 connectivity between a pair of neurons as displayed by GLMCC: **a** The GLMCC between units 924 (reference neuron j) and unit 923 (target neuron i). From this example of our calculations, we can imply that unit 924 has an excitatory connection with unit 923 , while it is also apparent that unit 923 has an inhibitory affect on unit 924. **b** The GLMCC between units 923 (reference neuron j) and unit 917 (target neuron i). In this example we can also see the excitatory connection, but this time it is in the opposite direction, as the target neuron (i) has an excitatory connection with the reference neuron(j). In the other half of the histogram, we have an example of an insignificant inhibitory connection, which ends up as a 0 value in the connectivity matrix.

Connectivity matrix

Connections between all units in our 132-neurons database are shown in Figure 4. In the first analysis, units were grouped into excitatory- and inhibitory-oriented neurons. Excitatory-oriented neurons were lined up first (left to right) and inhibitory-oriented neurons were then attached, ending up with 132 x 132 matrix divided into four sections as explained in Figure 4a. The 16 hours of recorded data was separated to eight 2-hour timeframes so that we could see the progression of the connectivity over time (Fig. 4b, Supplementary Fig. 1). The results match the background knowledge that during memory acquisition (0-3h) and consolidation (approx. 5-7h) phases, the connectivity pattern changes. In the first phase we can see an increase in the number of excitatory connections per neuron. In the second phase there is an increase in mean connectivity strength. In the second analysis, units were first divided into brain region subgroups based on their location on the NeuroPixel probe, as Units with depth below 1200µm were grouped to GC (n=93) and units with depth above 1200µm were grouped to S2 (n=39). Then, each subgroup was separately divided into

E/I groups, similar to the first analysis, resulting in a 132 x 132 matrix divided to 16 sections representing different connection types, as described in Figure 4c. Here as well the data was separated to two-hour timeframes as in the first analysis (Fig.4d, Supplementary Fig 1). Further analysis of these results show that GC-GC connections resemble the general pattern of the whole neuron ensemble activity in terms of connections per neuron, and thus might have a more significant effect on the ensemble activity in the process of CTA formation than S2-S2 connections. In both brain regions there are more excitatory than inhibitory connections per neuron.

Figure 4

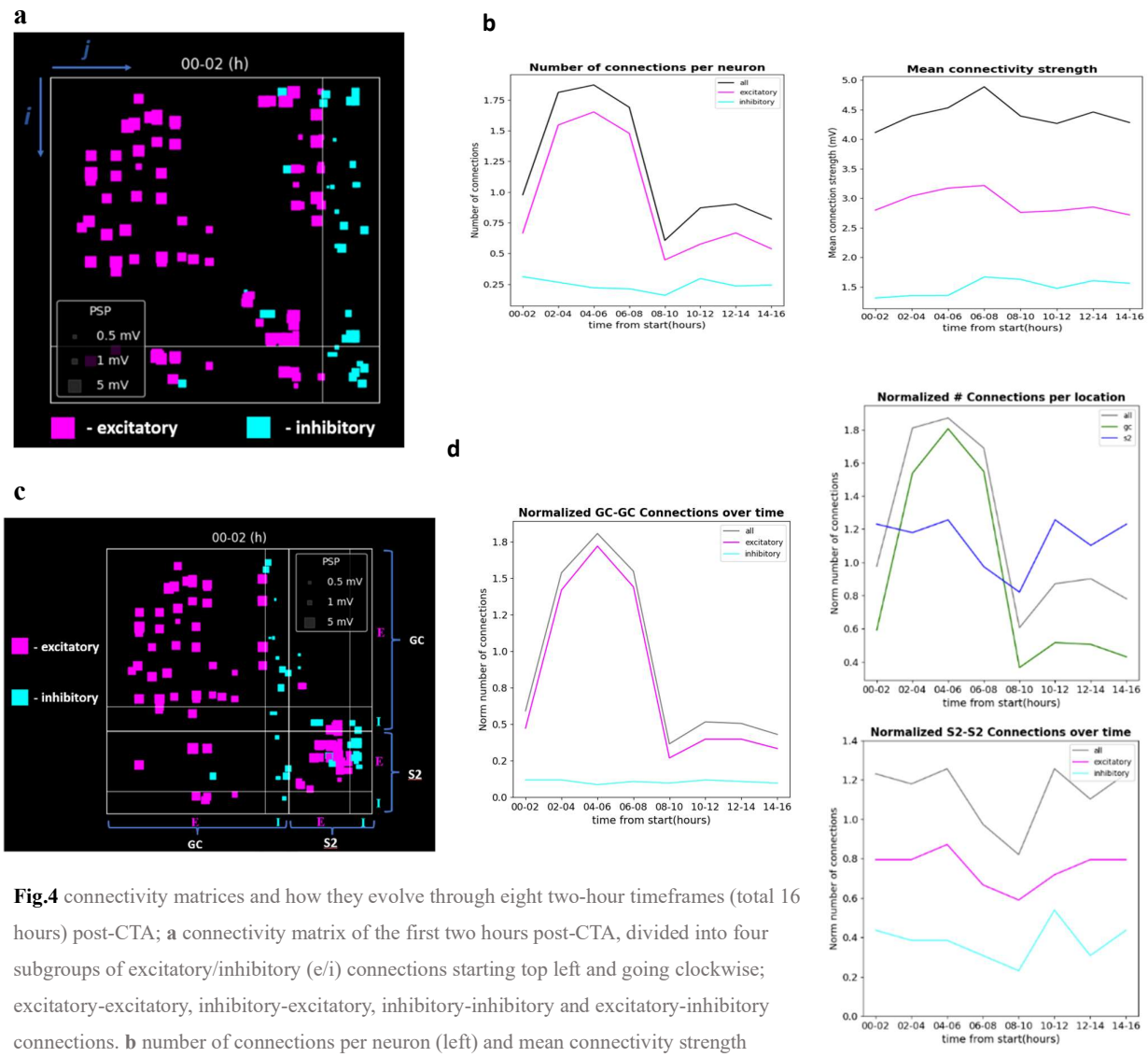


Fig.4 connectivity matrices and how they evolve through eight two-hour timeframes (total 16 hours) post-CTA; **a** connectivity matrix of the first two hours post-CTA, divided into four subgroups of excitatory/inhibitory (e/i) connections starting top left and going clockwise; excitatory-excitatory, inhibitory-excitatory, inhibitory-inhibitory and excitatory-inhibitory connections. **b** number of connections per neuron (left) and mean connectivity strength (right) over time. **c** connectivity matrix of the first two hours post-CTA, divided into 16 subgroups that differ in location (GC/S2) and orientation (excitatory/inhibitory) as mentioned in the axes. **d** number of connections per neuron between units located in the GC and their counterparts (left), number of connections per neuron between units located in S2 and their counterparts (right bottom) and number of connections per neuron in each specified location GC/S2 and altogether (top right).

Discussion

In this project we showed how to estimate connectivity of neuron ensembles in free-behaving animals using principles from Kobayashi's GLMCC method. To do so, we used data from one female rat's brain during the first 16 hours following CTA. The data was recorded from the rat's GC and S2 brain areas. The main goal was to develop a primary approach to analyze such data using GLMCC method, in a way that can estimate the network activity in a neuron ensemble throughout the process of CTA. To achieve this, we used principles and algorithms from Kobayashi's work and applied it with our data to generate connectivity matrices that can be manipulated, compared and statistically analyzed to find and demonstrate the dynamics of neuron ensemble connectivity throughout CTA process.

The connectivity matrices and time-course analysis allowed us to identify the specific connections between neurons in the gustatory cortex and somatosensory S2 regions. By grouping neurons based on their excitatory or inhibitory likelihood and their brain region locations, we gained insights into how different neuron populations contributed to the overall network activity during CTA.

From the results of the study, we could observe that the number of excitatory connections per neuron increased in both brain regions (GC and S2) during the memory acquisition phase (0-3 hours) while the mean connection strength increased during consolidation phase (5-7 hours). These findings match the background knowledge regarding these stages of long-term memory formation. Additionally, we found that the connectivity pattern differs within different brain regions (GC and S2). While connectivity patterns between GC neurons resemble that of the general population, S2 neurons show a much different and more stable activity over time. This may indicate that neurons in the gustatory cortex are primarily responsible for conveying information about the sensed flavour, while neurons in S2 may filter and process this information onwards into other brain regions based on past experiences.

The use of the GLMCC method proved to be an effective approach to estimate connectivity in neuron ensembles. This statistical tool allowed us to differentiate true connections from the effects of surrounding neurons, providing accurate and reliable results which matched the background knowledge. That being said, this method does not take into account bursting neurons. Bursting neurons are neurons that are capable of firing multiple rapid action potentials in a short period, followed by a period of reduced firing. This mechanism can be used to enhance signal transmission and processing, encode information more effectively and synchronize the activity of neuron ensembles. One method for reducing the effects of bursting on the data is deconvolution (Spark et al., 2022)[24], which can also be combined with GLMCC. While this property seems to be imperial to the research of network connectivity, in this study we chose not to address it since the majority of our data came from neurons that did not seem to be bursting neurons. This is also apparent since previous studies on bursting neurons rarely mention the GC as a brain region with many bursting neurons (such as the Hippocampus and the Thalamus)[29,30].

This study also faces some other limitations. The research was conducted on data from one female rat, which might limit the generalizability of the findings. Additionally, the used data came only from the first several hours post-CTA, while the neuronal activity before and further after the process could also have an influence on the result and lead to better insights. To strengthen the results, future studies should include a larger sample size and include data from before and further after the first hours following the manipulation.

Overall, in this project we have developed a primary approach, as well as algorithms, for using Cross-Correlation analysis on hours of recorded data that estimates and demonstrates how neuron ensembles connectivity changes over time. These foundations pave the way for future research in the field of neural mechanisms of behaviour such as learning and memory, using modern data acquisition techniques and advanced data analysis methods to explore the complexities of brain network connectivity.

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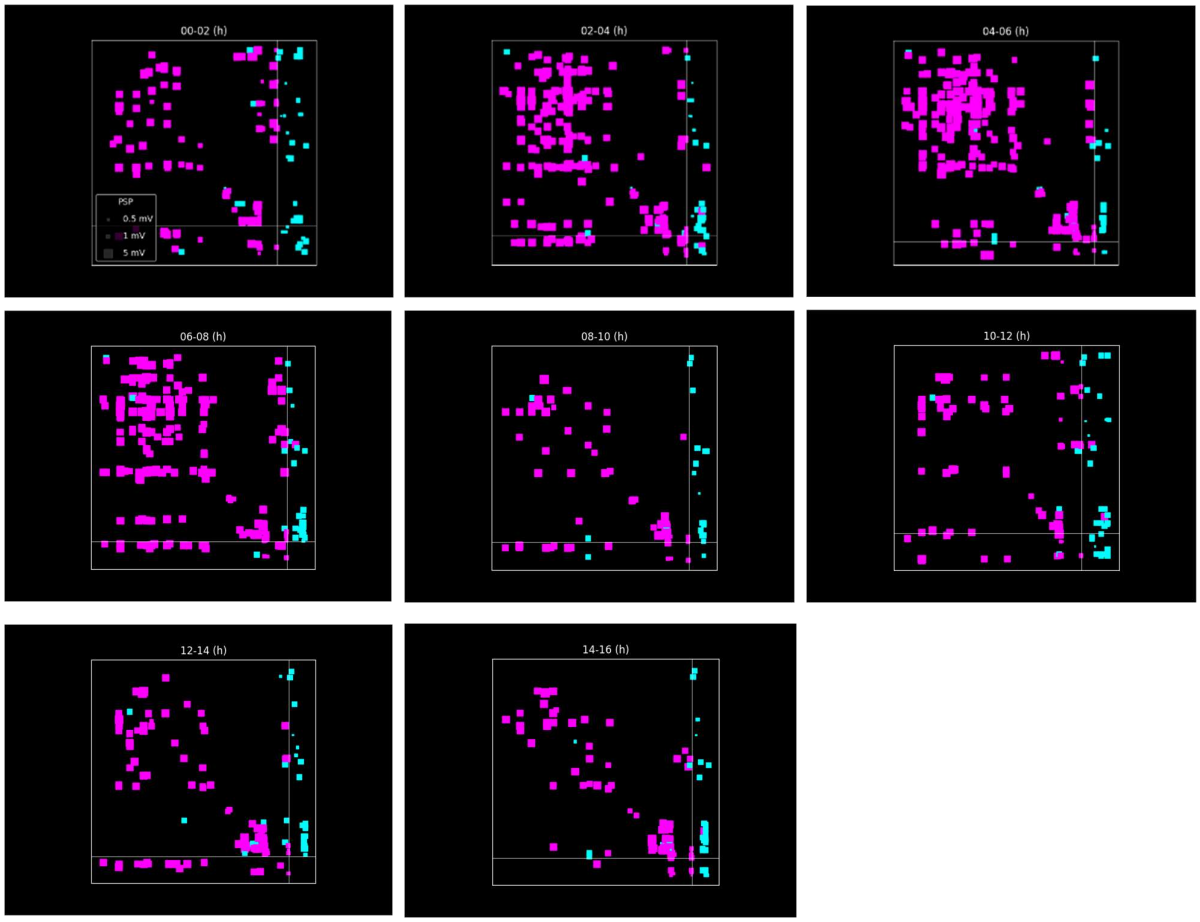
Supplements

Supplementary Figure 1: E/I connectivity matrices from all eight two-hour timeframes.



Supplementary Figure 2: GC/S2-E/I connectivity matrices from all eight two-hour timeframes